Phytotoxicity of equisetin and *epi*-equisetin isolated from *Fusarium equiseti* and *F. pallidoroseum*

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Fusarium equiseti and *F. pallidoroseum* are frequently reported as secondary colonizers of plant tissues. In this study they were isolated from the embryos of weathered cottonseed. Most isolates tested produced equisetin, an antibiotic, when grown on potato dextrose agar, rice, surface-sterilized cottonseed, or autoclaved cottonseed. This is the first report of equisetin from *F. pallidoroseum*. Equisetin was extracted from cultures of *F. equiseti* and *F. pallidoroseum* with acetone and dichloromethane, and partially purified by TLC. Two epimers of equisetin, designated as EQ and *epi*-EQ, were separated by HPLC. EQ or *epi*-EQ at 2·5–10 μg ml^{-1} suppressed germination or inhibited growth of various monocotyledonous and dicotyledonous seed, when the seed were incubated at 30 °C under aqueous shake conditions. The two epimers also inhibited the growth of young seedlings and caused necrotic lesions on the roots, cotyledons, and coleoptiles of tested plant seedlings. The results suggest that equisetin may be a pathogenic factor of *F. equiseti* and *F. pallidoroseum* on seed and seedling health of cotton and other plants.

Fusarium equiseti (Corda) Sacc. and *Fusarium pallidoroseum* (Cooke) Sacc. (syn. *Fusarium semitectum* auct. non Berk. & Rav.) are commonly found in warm temperate, subtropical, and tropical regions and infect a number of monocotyledonous and dicotyledonous plants (Booth, 1971; Farr *et al*., 1989). These two soil-borne fungi are generally considered to be secondary colonizers of plant tissues. On their own, however, they have been shown to cause diseases of importance in a variety of crops, e.g., snap bean (Dhingra & Muchovej, 1979), banana (Jones, 1991), tomato (Nedumaran & Vidyasekaran, 1981), cantaloup (Carter, 1979) and muskmelon (Adams, Gubler & Grogan, 1987).

The metabolite equisetin was first purified from maize grit medium cultures of *F. equiseti* strain NRRL 5537 (Burmeister *et al*., 1974) but it has not previously been reported in cultures of *F. pallidoroseum* or other *Fusarium* species. The structure of equisetin has been determined (Phillips *et al*., 1989), and it has been synthesized and its stereochemistry established (Turos, Audia & Danishefsky, 1989). Equisetin exists as two epimers that we refer to as EQ and *epi*-EQ (Fig. 1). EQ is the most widely studied of the two and Phillips *et al*. (1989) were the first to show that EQ is converted to *epi*-EQ.

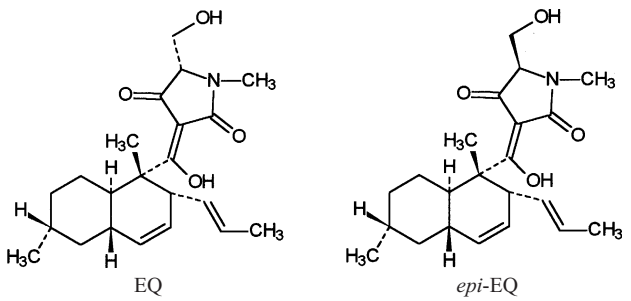

Fig. 1. Structures of equisetin (EQ) and *epi*-equisetin (*epi*-EQ).

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Equisetin has been shown to have strong antibiotic activity to certain Gram-positive bacteria (Burmeister *et al*., 1974; Burmeister, 1976), and its role as a possible human leukaemogen has been postulated (Phillips *et al*., 1989; Turos *et al*., 1989). Equisetin has not, however, previously been shown to be phytotoxic or to have other effects on plant tissues.

In the current study, we extracted and purified equisetin from cultures of several isolates of *F. equisti* and *F. pallidoroseum*, which were originally isolated from the embryos of weathered cottonseed. These fungi are often found in cottonseed after boll opening and weathering and are believed to be important pathogens, contributing to cottonseed deterioration (Roncadori, McCarter & Crawford, 1971; Klich, 1986; Wheeler, 1993). We describe the isolation of EQ and *epi*-EQ from cultures of both *F. equiseti* and *F. pallidoroseum*. We also describe the adverse effect of the two epimers on the germination and growth of seed and seedlings of various plants.

MATERIALS AND METHODS

Fungi

F. equiseti and *F. pallidoroseum* were isolated from cottonseed embryos of the cotton cvs Deltapine 14 (selection TM-1),

Deltapine 50, and TAMCOT HQ-95. The embryos were from cottonseed that had been weathered (deteriorated) under field conditions at College Station, TX, U.S.A. for 90–120 d after boll opening. Following harvest, the cottonseed was delinted with concentrated H_2SO_4, rinsed for 10 min with water, soaked in saturated $CaCO_3$ solution to neutralize any remaining acid, rinsed for 5 min with water, and dried overnight at 40 °C. The embryos were obtained by removing seedcoats and nucellar membranes from cottonseed that had been soaked in deionized water for 2–4 h. The embryos were surface-sterilized in a solution of 2% sodium hypochlorite and 0·001% (v/v) Triton X-100 (Sigma, St Louis, MO, U.S.A.) for 2 min with agitation; they were then rinsed three times in sterile, deionized water and placed on potato dextrose agar (PDA) amended with the antibiotics penicillin-G (50 mg l^{-1}), streptomycin sulphate (100 mg l^{-1}), tetracycline (50 mg l^{-1}), and erythromycin (35 mg l^{-1}). The embryos on PDA were incubated for 5–7 d at 25°. Fungi, believed to be either *F. equiseti* or *F. pallidoroseum*, were transferred to carnation leaf agar (CLA) after single spore isolation, and the pure cultures identified to *F. equiseti* or *F. pallidoroseum* on CLA according to Nelson, Toussoun & Marasas (1983). The fungi were maintained on CLA. Within each species, the isolates of *F. equiseti* or *F. pallidoroseum* used, differed morphologically or in terms of the metabolites produced as seen by thin-layer-chromatography (TLC).

Equisetin production on rice, PDA and cottonseed

Fernbach flasks (2·8 l), containing 100 g of long-grain rice and 100 ml of deionized water, were autoclaved for 30 min at 121°, and the moist rice was inoculated with 10 ml of macerate from cultures of *F. equiseti* or *F. pallidoroseum*. The inoculum was prepared in a blender by macerating fungal material from a 7–10 d old PDA culture (100 × 15 mm Petri dish) in 60 ml of sterile water. PDA and autoclaved or surface-sterilized cottonseed were also used as substrates for equisetin production after inoculation with macerates of *F. equiseti* and *F. pallidoroseum*. For autoclaved cottonseed, Fernbach flasks containing 100 g Deltapine 90 cottonseed and 100 ml of water were autoclaved twice for 20 min with a 24 h delay between sterilization steps to help kill resistant spore-forming microorganisms. For surface-sterilized cottonseed, 100 g of high quality Deltapine 90 cottonseed were placed in 1 l sterile Erlenmeyer flasks and agitated in a solution of 2% sodium hypochlorite and 0·001% Triton X-100 for 2 min. The seed were then rinsed three times in sterile water and soaked in 100 ml of sterile water for 6 h before being inoculated with *F. equiseti* or *F. pallidoroseum*. The PDA (20 ml) in 100 mm diam. Petri dishes was covered with 0·5 ml of macerate and the autoclaved and surface-sterilized cottonseed were inoculated with 10 ml of macerate. Both *Fusarium* species were grown on PDA, rice, or cottonseed for 8–12 d in the dark at 25° before extracting equisetin.

Isolation of a mixture of EQ and epi*-EQ by TLC*

The rice, PDA, and cottonseed cultures were covered with approximately two times the volume of PDA, rice or seed with acetone and agitated at 200 rpm on a rotary shaker at 25° for 8 h. A second acetone extraction was carried out for 8 h and the combined extracts were filtered through Whatman No. 3 filter paper. The filtrate was evaporated under vacuum to remove acetone, and the remaining aqueous solution was extracted twice with dichloromethane. The dichloromethane fractions were combined, dried over Na_2SO_4, and roto-evaporated to a residue under vacuum. The residue was dissolved in ethyl acetate and streaked on TLC plates coated with Baker G/HR (J. T. Baker, Phillipsburgh, NJ, U.S.A.), containing 2·5% (w/w) zinc silicate phosphor (Sigma, St Louis, MO, U.S.A.). The plates were developed in chloroform-acetone-formic acid (89·5 : 10 : 0·5, v/v/v, TLC system 1). The band containing EQ and *epi*-EQ was distinguished by its purple colour under 254 nm uv light and the pink colour produced when sprayed with 1% ferric chloride. The single band containing the two epimers was scraped off and extracted with ethyl ether-ethyl acetate-ethanol (12 : 12 : 1, v/v/v). This solutioin was rotoevaporated to dryness. Where necessary, additional purification of the EQ and *epi*-EQ mixture was carried out on silica gel plates developed with ethyl acetate-hexanes (bp 68–70°)-formic acid (69 : 30 : 1 v/v/v, TLC system 2). This TLC step was needed to remove cottonseed metabolites that interferred with the HPLC purification of *epi*-EQ.

Purification of EQ and epi*-EQ by HPLC*

Residues from TLC systems 1 or 2 were dissolved in methanol or acetonitrile (ACN) and initially analysed for EQ and *epi*-EQ on a Hewlett-Packard (HP) 1090 liquid chromatograph equipped with a diode array detector and HP Vectra Chemstation operating system. Separations were achieved using a Phenomenex Hypersil-5-C18 (250 × 4 mm) column, maintained at 40° in conjunction with a mobile phase of 80% ACN and 20% water (both with 0·07% H_3PO_4) run isocratically at a flowrate of 1·25 ml min^{-1} for 15 min (HPLC system 1). The column effluent was monitored at 254 nm.

EQ was purified using a HP 1050 liquid chromatograph attached to a ISCO Foxy-200 fraction collector. Samples (100 µl) were injected onto a Phenomenex Hypersil-5-C18 (250 × 10 mm) column maintained at ambient temperature and eluted using a mobile phase of ACN and water (both with 0·07% H_3PO_4) under a gradient. In this gradient, a mix of 80% ACN and 20% water was run at a flowrate of 2·75 ml min^{-1} for 17·5 min in order to separate and elute EQ and its neighbouring compound *epi*-EQ. The ACN content and flowrate were increased to 100% and 4·5 ml min^{-1}, respectively, for 6·5 min to clean the column of any sample impurities before the next injection (HPLC system 2). The collected EQ and *epi*-EQ fractions were each rotoevaporated until the volume was decreased by one-third, extracted into ethyl acetate and then rotoevaporated to dryness.

Plants used in bioassays

Brassicaceae: *Brassica napus*, rape, cv. Dwarf Essex; *B. oleracea*

var. *botrytis*, broccoli, cv. Waltham 29; cauliflower, cv. Snowball Improved; *B. oleracea* var. *capitata*, cabbage, cv. Early Jersey Wakefield; *B. oleracea* var. *gemmifera*, brussels sprouts, cv. Long Island, and *B. rapa*, turnip cv. Purple Top White Globe. Cucurbitaceae; *Cucumis sativus*, cucumber cv. Sumter. Malvaceae: *Gossypium hirsutum*, cotton, cv. TAMCOT HQ-95. Poaceae: *Oryza sativa*, upland rice, cv. Lemont, and *Secale cereale*, rye. Solanaceae: *Lycopersicon esculentum*, tomato, cv. Early Girl.

Seed and seedling bioassays with equisetin

Seed and seedlings of various plants were treated with EQ or *epi*-EQ in liquid shake cultures by the method of Kuboi & Fujii (1984). Seedlings were treated with the two epimers when their combined root and shoot length was 1–5 mm. Most plants used in the studies were grown for 18 h before being exposed to the two epimers, but cotton and cucumber were exposed at 24 h and rice and tomato were exposed at 56 h.

Unless otherwise stated, 10 seeds or 10 seedlings were placed in 125 ml Erlenmeyer flasks with 25 ml deionized water containing 1–10 μg ml^{-1} EQ or *epi*-EQ, which were added to the water in 0·125 ml of methanol. The cultures containing seed were then incubated in the dark at 30° on a rotary shaker at 80 rpm for 4 or 5 d, and those containing seedlings were incubated for 3 or 4 d. Cotton, cucumber, rice and tomato were usually incubated for a day longer than the other plants, so they would be of desired size. For controls, 0·125 ml of methanol was added in place of the epimers dissolved in methanol. Experiments with seed and seedlings were repeated on three or more occasions. In experiments with cottonseed, Fernbach flasks containing 500 ml of water with 15 cottonseed were used. The cottonseed was shaken in water without EQ for 24 h to remove abscissic acid and start germination. The water was discarded and EQ in 2·5 ml of methanol was added to 500 ml of fresh water. The EQ-treated cultures were then shaken for 4 d at 100 rpm in the dark at 30°. For controls, 15 cottonseeds were shaken under the same conditions in 500 ml of water, containing 2·5 ml of methanol without EQ.

RESULTS

Isolation of fungi from cottonseed

F. equiseti and *F. pallidoroseum* were frequently isolated from seed and embryos that had been weathered for 90–120 d after boll opening. Other fungi frequently recovered from cottonseed and cottonseed embryos included *Alternaria* spp. (believed to be *A. alternata*), *Cladosporium* spp. and *Fusarium chlamydosporum*. During a period of 2 years, over 25% of the embryos were infected, and each of the infected embryos contained one to three fungi. In a typical study during 1993, for example, 87% of the seed of Deltapine 50 and 38% of the embryos were infected with fungi 90 d after boll opening. In this study, 37·5 and 8·5% of 400 whole seed were infected with *F. equisiti* and *F. pallidoroseum*, respectively, versus 26·5 and 6·5% of 400 embryos.

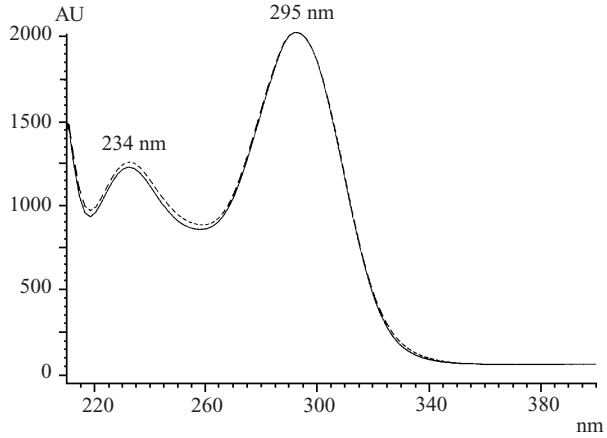


Fig. 2. UV-visible spectra of EQ and *epi*-EQ, taken using HPLC and a diode array detector. Dashed line = EQ; solid line = *epi*-EQ. HPLC system 1.

Table 1. EQ production from rice cultures of *F. equiseti* and *F. pallidoroseum*[a]

	EQ (% of total equisetin)
F. equiseti	
B21	88
B24	89
B25	91
B27	92
B28	93
B30	95
B37	90
B39	90
B40	91
B85	92
F. pallidoroseum	
B2	84
B3	91
B10	86
B11	91
B16	94
B22	88
B46	85
PS-11	91

[a] Cultures were grown on rice for 12 d at 25° and extracted with dichloromethane. The extracts were then dried under vacuum and the residues dissolved in acetonitrile for analysis by HPLC. % *epi*-EQ = 100 − % EQ.

Equisetin production by F. equiseti *and* F. pallidoroseum

Eight of eleven isolates of *F. pallidoroseum* and nineteen of twenty isolates of *F. equiseti* from cottonseed embryos produced equisetin on rice. Isolates of the two fungi also produced equisetin on PDA and on autoclaved or surface sterilized cottonseed. Equisetin production was greatest on rice. Rice became the preferred substrate in our experiments because it lacked compounds that interfere with the chromatographic purification of EQ and *epi*-EQ by TLC and HPLC.

Purification and characterization of equisetin

The material identified as equisetin (EQ and *epi*-EQ) had TLC

Table 2. Effect of EQ on root and shoot length of various plants[a]

	EQ treatment (μg ml^{-1})	Root length (mm)	Relative root growth (% of control)	Shoot length (mm)	Relative shoot growth (% of control)
Broccoli	0	32·1 ± 3·6 A	100	14·3 ± 1·2 A	100
	2·5	6·0 ± 0·6 B	19	14·4 ± 1·1 A	101
	10	1·5 ± 0·1 C	5	8·4 ± 0·6 B	59
Brussels Sprouts	0	37·9 ± 2·4 A	100	19·3 ± 1·8 A	100
	2·5	8·7 ± 0·9 B	23	14·6 ± 1·9 A	76
	10	1·6 ± 0·1 C	4	7·7 ± 1·1 B	40
Cabbage	0	33·5 ± 1·1 A	100	13·8 ± 1·1 A	100
	2·5	9·9 ± 0·9 B	30	13·3 ± 1·0 A	96
	10	2·0 ± 0·2 C	3	6·7 ± 0·5 B	9
Cauliflower	0	22·7 ± 3·6 A	100	12·1 ± 1·3 A	100
	2·5	7·2 ± 0·6 B	32	8·4 ± 0·7 B	69
	10	2·4 ± 0·1 C	11	3·9 ± 0·6 C	32
Cucumber	0	43·5 ± 3·3 A	100	16·8 ± 1·3 A	100
	2·5	23·9 ± 1·4 B	55	13·4 ± 1·3 B	80
	10	4·2 ± 0·2 C	10	7·5 ± 0·6 C	45
Raddish	0	54·9 ± 6·3 A	100	19·4 ± 1·7 A	100
	2·5	27·0 ± 5·6 B	49	14·7 ± 1·6 B	76
	10	3·2 ± 0·3 C	6	6·6 ± 0·7 C	34
Rape	0	45·3 ± 5·0 A	100	16·8 ± 2·2 A	100
	2·5	9·8 ± 1·5 B	22	8·2 ± 1·5 B	49
	10	2·6 ± 0·3 C	6	3·5 ± 0·3 C	21
Tomato	0	40·2 ± 3·5 A	100	11·7 ± 1·6 A	100
	2·5	7·9 ± 1·0 B	20	5·6 ± 0·8 B	48
	10	2·2 ± 0·2 C	6	4·5 ± 0·6 B	39
Turnip	0	54·3 ± 4·4 A	100	24·6 ± 2·0 A	100
	2·5	10·5 ± 0·9 B	19	16·8 ± 2·1 B	68
	10	1·7 ± 0·1 C	3	4·6 ± 0·5 C	19
Rice	0	25·1 ± 1·2 A	100	21·1 ± 0·6 A	100
	2·5	8·6 ± 0·8 B	34	8·9 ± 0·6 B	42
	10	1·9 ± 0·6 C	8	2·6 ± 0·5 C	12
Rye	0	25·6 ± 2·2 A	100	14·2 ± 1·1 A	100
	2·5	18·4 ± 1·7 B	72	9·9 ± 0·8 B	70
	10	0·5 ± 0·3 C	2	2·6 ± 0·7 C	18

[a] Values for root and shoot lengths are the mean ± S.E. Data were subjected to analysis of variance. Means within each plant species not showing a letter in common are significantly different ($P < 0·05$) by Fisher's least-significant-difference test. Tomato ($n = 9$); others ($n = 10$).

properties identical to those of an authentic reference sample of EQ provided by Dr R. F. Vesonder (Northern Regional Research Laboratory, ARS, USDA, Peoria, IL, U.S.A.). It appeared as a single band in TLC systems 1 and 2 with R_t values of 0·27 and 0·43, respectively. HPLC system 1 resolved the equisetin mixtures into two components with retention times of 5·5 and 6·0 min. The retention time and uv-visible spectrum of the first component (5·5 min) was identical to that of the authentic sample of EQ. The uv-visible spectrum of the second component (6 min) was almost identical to that of EQ (Fig. 2). The mixed sample was separated into two fractions by preparative HPLC (HPLC system 2). The first fraction, corresponding to the first peak, yielded a pale pink non-crystalline solid consisting of 99% EQ and 1% *epi*-EQ. The second fraction, containing the tailend of the first peak and all of the second peak, gave a dark pink viscous liquid. This second fraction was composed mostly of *epi*-EQ with variable amounts of EQ. The second fraction was further purified by the same preparative HPLC system until a pale pink non-crystalline solid consisting of greater than 95% *epi*-EQ and less than 5% EQ was obtained.

Table 1 gives the percentages of EQ relative to total equisetin (EQ + *epi*-EQ) that were determined using HPLC system 1 with extracted material from 12 d-old rice cultures of various *F. equiseti* and *F. pallidoroseum* isolates. Similar levels of EQ, ranging from 87·9–97·8% were obtained when *F. equiseti* isolates B27, B37 and B85 Table 1 and *F. pallidoroseum* B3, B11 and B22 were grown for 12 d on PDA and on autoclaved and surface-sterilized cottonseed (data not shown).

The presence of EQ in *F. equiseti* and *F. pallidoroseum* was confirmed with nuclear magnetic resonance (NMR) spectroscopy. EQ, isolated from cultures of the two fungi, had a carbon-13 NMR spectrum [^{13}C-NMR (CDC_{13}, 25°) δ 199·0 ($C_{4'}$), 190·5 (C_1), 177·1 ($C_{2'}$), 130·9 (C_5 or C_{13}), 130·0 (C_5 or C_{13}), 127·0 (C_4 or C_{14}), 126·6 (C_4 or C_{14}), 100·1 ($C_{3'}$), 66·7 ($C_{5'}$), 60·5 ($C_{6'}$), 48·7 (C_2), 45·0 (C_3), 42·2 (C_7), 39·9 (C_{11}), 38·6 (C_6), 35·7 (C_9), 33·5 (C_8), 28·3 (C_{10}), 27·3 ($C_{7'}$), 22·5 (C_{16}), 17·9 (C_{15}), 14·0 C_{12}] almost identical to that obtained for EQ at −20° by Phillips *et al.* (1989).

We found that treatment of EQ with pyridine for 4 d produced a mixture of 80% EQ and 20% *epi*-EQ. This is consistent with a report by Phillips *et al.* (1989) who showed

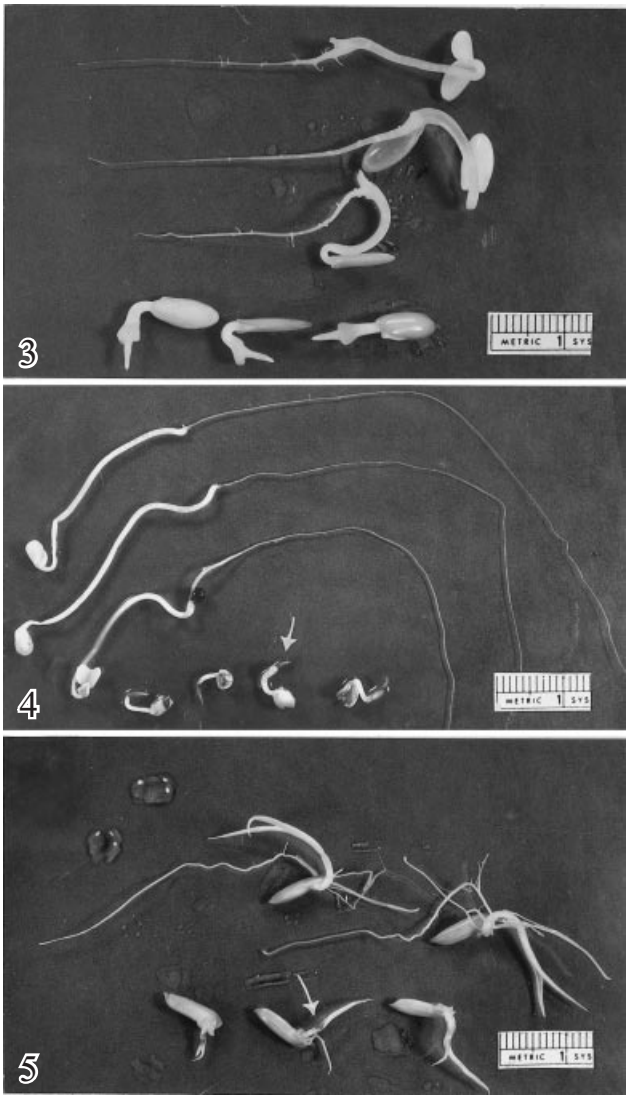
that pyridine converted EQ to a mixture of 50% EQ and 50% *epi*-EQ *via* an epimerization reaction. We also found that EQ gradually epimerized to *epi*-EQ when left in contact with uninoculated rice medium over a 28 day period. In a typical experiment, we treated moist rice with a mixture of 96% EQ and 4% *epi*-EQ initially. EQ and epi-EQ levels were measured at 2, 9, 12 and 28 d and found to contain 94·6, 93·7, 93·5 and 91·6% EQ and 5·4, 6·3, 6·5 and 8·4% *epi*-EQ, respectively. This showed that EQ had epimerized to *epi*-EQ without *F. equiseti* or *F. pallidoroseum* being present. Similar results also were obtained with inoculated rice. In 5-d-old rice cultures of *F. pallidoroseum* (B22), *epi*-EQ was 0·8% and in 12-d-old cultures it was 4·6%. Similarly 5-d-old rice cultures of *F. equiseti* (B85) contained 1·3% *epi*-EQ and 12-d-old cultures contained 2·8% *epi*-EQ.

Phytotoxicity of equisetin to seed and seedlings

Seedlings were preferred over seed for bioassays with EQ and *epi*-EQ because they were known to be viable and could easily be arranged in similar groups by size. EQ inhibited the growth of seedlings of various dicotyledonous and monocotyledonous plants. Root, shoot and coleoptile growth was usually inhibitied by EQ at 2·5–10 μg ml^{-1} (Table 2). EQ at 10 μg ml^{-1} caused necrotic lesions on roots, cotyledons, or coleoptiles of various types of seedlings (Figs 3–5). Studies with cucumber, cotton, rape, rice, and turnip showed that after seedlings were treated with 10 μg ml^{-1} EQ for 18 h they did not resume growth when placed in water without EQ for an additional 72 h. This indicated that the seedlings were permanently damaged by EQ. Tables 2 and 3 and Figs 3–5 show the inhibitory effects of EQ on various types of seedlings.

When seed of cucumber, rape, rice, and turnip were treated with EQ at 1–10 μg ml^{-1}, EQ inhibited seedling growth but often failed to prevent germination even at the highest equisetin level of 10 μg ml^{-1}. The inhibitory effect of EQ on shoot length was usually less than that on root length, and with cucumber, rape, rice, and turnip, 10 μg ml^{-1} EQ severely inhibited the growth of roots. Seedlings from seed, treated with 10 μg ml^{-1} EQ, were similar in appearance to those from 18–56 h-old seedlings treated with the same concentration. In both cases, after treatment with 10 μg ml^{-1} EQ, the seedlings of all four plants developed severely stunted roots, and with rape, rice and turnip the root tips were visibly necrotic. In a typical study, using 1, 2·5, 5 and 10 μg ml^{-1} EQ, inhibition of total seedling growth occurred as follows: cucumber seed, 20, 28, 43 and 69%, respectively; rape seed, 0, 14, 64 and 83%, respectively; rice, 16, 30, 67 and 91% respectively; and turnip seed, 18, 35, 84 and 92%, respectively.

epi-EQ was tested with cucumber, rice, and turnip seedlings and also was found to be phytotoxic. The effects of *epi*-EQ and EQ on the growth of 18-h old turnip seedlings were compared after the seedlings were treated for 72 h. The two isomers had a similar inhibitory effect on growth of the seedlings at concentrations of 2·5 or 10 μg ml^{-1} (Table 4). Also, at 10 μg ml^{-1} root tips of the treated seedlings were damaged. In these studies, *epi*-EQ at 10 μg ml^{-1} also strongly inhibited the growth of seedlings from cucumber, rice and turnip seed (data not shown). The results with *epi*-EQ and EQ indicated that the two epimers were similar in phytotoxicity.



Figs 3–5. Seedlings from bioassays with 10 μg ml^{-1} EQ (bottom) and control (top). Note lack of root growth in all three treated plants, and necrotic lesions (arrows). **Fig. 3.** Cucumber. **Fig. 4.** Rape. **Fig. 5.** Rice.

Table 3. Effects of EQ on seedling length of cotton

EQ[b] treatment (μg ml^{-1})	Seedling length (mm)	Relative growth (% of control)
0	59·1 ± 4·9 A[a]	100
2·5	41·9 ± 2·9 B	71
5	26·5 ± 2·3 C	45
10	11·0 ± 0·6 D	19

[a] Mean ± s.e. ($n = 15$). Data were subjected to analysis of variance. Means not showing a letter in common are significantly different ($P < 0·05$) by Fisher's least-significant-difference test.

[b] EQ was added to 500 ml of deionized water in 2·5 ml of methanol. Ten 24 h-old cotton seedlings were incubated at 30° for 96 h at 80 rpm.

Table 4. Effect of EQ and *epi*-EQ on root and shoot length of turnip seedlings[a]

Equisetin[b] Isomer	Equisetin concn (μg ml^{-1})	Root length (mm)	Relative root growth (% of control)	Shoot length (mm)	Relative shoot growth (% of control)
None	0	42·6 ± 4·9 A	100	18·8 ± 2·1 A	100
EQ	2·5	17·8 ± 2·0 B	42	22·8 ± 1·9 A	121
epi-EQ	2·5	15·3 ± 2·3 B	36	13·2 ± 1·5 B	70
None	0	46·0 ± 2·3 A	100	15·7 ± 1·5 A	100
EQ	10	3·2 ± 0·3 B	7	4·8 ± 0·3 B	31
epi-EQ	10	3·5 ± 0·3 B	8	3·6 ± 0·2 B	23
None	0	50·8 ± 3·2 A	100	22·2 ± 1·1 A	100
EQ	10	3·5 ± 0·3 B	7	7·6 ± 1·2 B	34
epi-EQ	10	4·0 ± 0·2 B	8	4·5 ± 0·4 C	20

[a] Values for root and shoot lengths are the mean ± S.E. Data were subjected to analysis of variance. Means within each study not showing a letter in common are significantly different ($P < 0·05$) by Fisher's least-significant-difference test. First set of treatments with EQ and *epi*-EQ ($n = 12$); second and third set of treatments with EQ and *epi*-EQ ($n = 10$).

[b] EQ and *epi*-EQ were added to 25 ml of deionized water in 0·125 ml of methanol.

DISCUSSION

Equisetin was previously found in cultures of *F. equiseti* (Burmeister *et al*., 1974; Phillips *et al*., 1989), but this is the first report of its isolation from *F. pallidoroseum*. In related studies, we also looked for equisetin in rice cultures of *Alternaria* spp., *F. chlamydosporum* and *F. monoiliforme* but did not find it. To our knowledge equisetin has not been found in the cultures of any other fungi and may be unique to *F. equiseti* and *F. pallidoroseum*.

This is also the first mention of the phytotoxicity of EQ and *epi*-EQ to seed and seedlings and the first report of the isolation and purification of EQ and *epi*-EQ by TLC and HPLC. The mode of action of EQ and *epi*-EQ on seed and seedlings is not known, but the two epimers may have an effect on energy-related reactions in mitochondria. Equisetin has previously been shown to affect a number of energy-related reactions in the photosynthetic bacterium *Rhodospirillum rubrum*, mitochondria of *Saccharomyces cerevisiae*, and rat liver mitochondria. In studies with chromatophores of *R. rubrum*, Nyren & Strid (1989) showed that equisetin inhibited light-induced proton uptake, light-induced carotenoid absorbance shift, photophosphorylation, and hydrolysis of ATP and inorganic pyrophosphate. With mitochondrial preparations of *S. cerevisiae*, Lundin *et al*. (1992) demonstrated that equisetin stimulated the hydrolysis of inorganic pyrophosphate by pyrophosphatase. Konig, Kapus & Sarkadi (1993) showed in studies with rat liver mitochondria, that equisetin specificly inhibited substrate anion carriers of the mitochondrial inner membrane. Konig *et al*. (1993) found that equisetin does not directly inhibit the electron transport chain in rat liver mitochondria but does prevent the mitochondrial transport of ATP, orthophosphate and succinate across the inner mitochondrial membrane. The studies with these three organisms suggest that EQ and *epi*-EQ may affect mitochondria in seed or young seedlings. Thus, since mitochondria heavily populate meristematic tissues, this would explain why in the present study the two epimers apparently had a detrimental effect on meristematic tissues and damaged root tips.

This is the first report of *epi*-EQ existing as a natural product, although Phillips *et al*. (1989) were the first to discover the compound and to show its relationship to EQ. Our results indicate that EQ is formed enzymically under culture conditions, while *epi*-EQ is the result of slow non-enzymic epimerization. We found EQ slowly epimerized to *epi*-EQ on uninoculated rice near pH 6. Also, we and Phillips *et al*. (1989) determined that the epimerization of EQ to *epi*-EQ occurred in pyridine solution. These studies show that *epi*-EQ is made from EQ under acid and basic conditions and that both epimers can be expected in older cultures of *F. equiseti* and *F. pallidoroseum*. The fact that *epi*-EQ was found in rice, PDA, and in autoclaved and surface-sterilized cottonseed cultures suggests that EQ and *epi*-EQ are likely to be produced on a wide variety of substrates.

In the present study EQ and *epi*-EQ were purified from cultures of *F. pallidoroseum* and *F. equiseti* which were obtained from the embryos of weathered cottonseed. Both of these fungi produced equisetin on surface-sterilized (non-autoclaved) cottonseed, showing that they were able to produce the compound while infecting living tissues. The two epimers of equisetin were found to be phytotoxic to a variety of plants during seed germination or during seedling growth. These results indicate that equisetin is produced by *F. equiseti* and *F. pallidoroseum* as they infect seed or tissues of plants and that EQ and *epi*-EQ may act as phytotoxins in infected seed or other plant tissues.

Weathered cottonseed frequently produces seedlings that are stunted or that have severely damaged root systems. The fact that equisetin-treated seed or young seedlings often produce plants with root systems that are similar in appearance to those from weathered seed, further suggests that equisetin may be involved in seed deterioration.

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